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ORIGINAL ARTICLE

Epidermal Growth Factor Receptor (EGFR) Pathway Biomarkers in the Randomized Phase III Trial of Erlotinib Versus Observation in Ovarian Cancer Patients with No Evidence of Disease Progression after First-Line Platinum-Based Chemotherapy

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Abstract

Background In this work, we aimed to identify molecular epidermal growth factor receptor (EGFR) tissue biomarkers in patients with ovarian cancer who were treated within the phase III randomized European Organisation for Research and Treatment of Cancer-Gynaecological Cancer Group (EORTC-GCG) 55041 study comparing erlotinib with observation in patients with no evidence of disease progression after first-line platinum-based chemotherapy.

Methods Somatic mutations in *KRAS*, *BRAF*, *NRAS*, *PIK3CA*, *EGFR*, and *PTEN* were determined in 318 (38 %) and expression of EGFR, pAkt, pMAPK, E-cadherin and Vimentin, and *EGFR* and *HER2* gene copy numbers in 218 (26 %) of a total of 835 randomized patients. Biomarker data were correlated with progression-free survival (PFS) and overall survival (OS).

Results Only 28 mutations were observed among *KRAS, BRAF, NRAS, PIK3CA, EGFR*, and *PTEN* (in 7.5 % of patients), of which the most frequent were in *KRAS* and *PIK3CA. EGFR* mutations occurred in only three patients. When all mutations were pooled, patients with at least one mutation in *KRAS*, *NRAS*, *BRAF*, *PIK3CA*, or *EGFR* had

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longer PFS (33.1 versus 12.3 months; HR 0.57; 95 % CI 0.33 to 0.99; P=0.042) compared to those with wild-type tumors. EGFR overexpression was detected in 93 of 218 patients (42.7 %), and 66 of 180 patients (36.7 %) had *EGFR* gene amplification or high levels of copy number gain. Fifty-eight of 128 patients had positive pMAPK expression (45.3 %), which was associated with inferior OS (38.9 versus 67.0 months; HR 1.81; 95 % CI 1.11 to 2.97; P=0.016). Patients with positive *EGFR* fluorescence in situ hybridization (FISH) status had worse OS (46.1 months) than those with negative status (67.0 months; HR 1.56; 95 % CI 1.01 to 2.40; P=0.044) and shorter PFS (9.6 versus 16.1 months; HR 1.57; 95 % CI 1.11 to 2.22; P=0.010). None of the investigated biomarkers correlated with responsiveness to erlotinib.
Conclusions In this phase III study, increased *EGFR* gene copy number was associated with worse OS and PFS in patients with ovarian cancer. It remains to be determined whether this association is purely prognostic or is also predictive.

1 Introduction

A major focus of cancer therapy research over the past decade has been in the targeting of cellular processes affecting cell proliferation, differentiation, growth, and survival. One of the best studied among these is the epidermal growth factor receptor (EGFR), given its dysregulation in the vast majority of human tumors of epithelial origin [1]. EGFR is a member of the ErbB family consisting of four tyrosine kinase (TK) receptors: EGFR/ErbB-1, HER-2/neu (ErbB-2), HER-3 (ErbB-3), and HER-4 (ErbB-4) [2]. Binding of specific ligands such as epidermal growth factor (EGF) and transforming growth factor α (TGF-α) to the EGFR results in the dimerisation of the receptor, tyrosine auto-phosphorylation, with subsequent initiation of the intracellular signaling pathways cascade. Downstream signaling pathways include the ras-raf-mitogen-activated protein kinase (Ras/Raf/MAPK) and the phosphatidylinositol 3-kinase (PI3K/AKT) pathways, which are all involved in cell proliferation and survival [2].

EGFR overexpression is observed in up to 98 % of advanced epithelial ovarian cancers (EOCs) [3, 4] and has been associated with a worse prognosis [3, 5], although data are conflicting [6, 7]. The targeting of EGFR or its downstream pathways, therefore, appears to be a promising strategy in EOC. Monoclonal antibodies (mAbs), such as cetuximab and panitumumab, bind competitively to the extracellular domain of EGFR, leading to internalization and degradation of the receptor, while tyrosine kinase inhibitors (TKIs) like erlotinib and gefitinib compete with ATP for binding to the receptor's intracellular TK domain, thereby inhibiting TK activity. Both molecular strategies have been investigated in EOC (Table 1).

Although several clinical prognostic factors have been identified in EOC (e.g., age at diagnosis, extent of disease, amount of residual disease after initial surgery, tumor grade, and tumor histological subtype) [17, 18], molecular prognostic markers, or even predictive biomarkers for targeted agents, are still lacking. In other disease entities such as non-small cell lung cancer (NSCLC) and metastatic colorectal cancer (mCRC)—where anti-EGFR therapies have been widely studied—several predictive biomarkers for anti-EGFR agents have been identified. EGFR protein expression as determined by immunohistochemistry (IHC) was the first biomarker claimed to predict a response in NSCLC. However, data have been conflicting, and its association with sensitivity to anti-EGFR therapies remains unclear [19–21]. Activating mutations in EGFR exons 18 to 21 [22–24] and an increased EGFR gene copy number may increase sensitivity to anti-EGFR agents [19, 20, 25–28], whereas deregulation of downstream targets of the EGFR pathway (i.e., mutations in the *KRAS*, *NRAS*, *BRAF*, or *PIK3CA* genes or loss of PTEN protein expression) have emerged as an important negative predictive factor for the efficacy of anti-EGFR mAbs [29–34]. However, following the initial response, NSCLC patients harboring activating EGFR mutations may become resistant to TKIs due to an acquired secondary EGFR kinase domain mutation, T790M [35]. While an increased HER2 gene copy number in NSCLC may affect sensitivity to EGFR TKIs [36–39], preclinical and clinical studies in mCRC have shown cetuximab resistance in cases with HER2 gene amplification [40, 41]. Additionally, in NSCLC, TKI responsiveness may be predicted by EGFR downstream proteins such as activated (phosphorylated) AKT [19]. Furthermore, epithelial-mesenchymal transition (EMT), a key player in cancer progression and metastasis, which is characterized by a loss in expression of E-cadherin and a gain in vimentin expression, is associated with resistance to gefitinib and erlotinib in NSCLC [42, 43]. Thus far, the extensive data investigating responsiveness to anti-EGFR agents have focused mainly on NSCLC and mCRC; data for ovarian cancer, on the other hand, are scarce.

Studies with EGFR-targeted agents in EOC suggest that only a subgroup of ovarian cancer patients might benefit from these therapies (Table 1). However, Gordon et al. reported 44 % stable disease in a phase II study in patients with refractory recurrent EGFR-positive epithelial ovarian cancer treated with erlotinib [8]. This finding led to the European Organisation for Research and Treatment of Cancer-Gynaecological Cancer Group (EORTC-GCG) 55041 trial, in which ovarian cancer patients with no evidence of disease progression following first-line platinum-based chemotherapy were treated with erlotinib as maintenance therapy. Unfortunately, in this unselected patient population, erlotinib did not improve progression-free or overall survival. The aim of the present explorative translational biomarker study based

Table 1 Clinical trials reporting administration of anti-EGFR agents to patients with epithelial ovarian cancer

Study	Treatment regimen	Phase	Patient population	No. of pts	Response
Erlotinib					
Gordon et al. [8]	150 mg/d	II	Recurrent EGFR-positive	34	PR: 2 (6 %) SD: 15 (44 %) PD: 17 (50 %)
Blank et al. [9]	Paclitaxel (175 mg/m^2) and carboplatin (AUC6) every 3 weeks and erlotinib 150 mg/d	II	Primary a: after optimal CRS, b: after suboptimal CRS, c: before CRS	56 a: 28, b: 23, c: 5	CR (a): 8 (29 %) CR (b): 3 (13 %)
Hirte et al. [10]	Carboplatin (AUC5) every 3 weeks and erlotinib 150 mg/d	II	Recurrent a: platinum-sensitive b: platinum-resistant	50 a: 33 b: 17	CR (a): 3 (9 %) PR (a): 14 (42 %) PR (b): 1 (6 %)
Gefitinib					
Schilder et al. [11]	500 mg/d	II	Recurrent	27	PR: 1 (3.7 %) PFS>6 m: 14.8 %
Posadas et al. [12]	500 mg/d	II	Recurrent	24	CR: 0 PR: 0 SD: 9 (38 %)
Wagner et al. [13]	Tamoxifen 40 mg/d and gefitinib 500 mg/d	II	Recurrent	56	SD: 16 (28.6 %)
Pautier et al. [14]	Paclitaxel (175 mg/m^2) and carboplatin (AUC5) every 3 weeks and gefitinib 500 mg/d	II	Recurrent a: platinum-resistant b: platinum-sensitive	68 a: 26 b: 42	CR (a): 1 (3.8 %) CR (b): 10 (23.8 %) PR(a): 4 (15.4 %) PR (b): 16 (38.1 %)
Cetuximab					
Schilder et al. [15]	Initial dose 400 mg/m^2 and 250 mg/m^2 weekly for two 3-week cycles	II	Recurrent	25	PR: 1 (4 %) SD: 9 (36 %)
Secord et al. [16]	Initial dose 400 mg/m^2, followed by weekly infusion of 250 mg/m^2 and carboplatin (AUC6) every 3 weeks	II	Recurrent platinum-sensitive EGFR positive	26	CR: 3 (11.5 %) PR: 6 (23 %) SD: 8 (30.8 %)

AUC area under the curve, *CR* complete response, *CRS* cytoreductive surgery, *No. of pts* number of evaluable patients, *PR* partial response, *SD* stable disease

on the prospective EORTC-GCG 55041 trial [44] was to determine the frequency of alterations of components of the EGFR pathways and to correlate biomarker data with the efficacy of erlotinib.

2 Materials and Methods

2.1 Patient Population

In the EORTC-GCG 55041 phase III trial, eligible patients were those with histologically confirmed high-risk FIGO stage I (grade 3, or aneuploid grade 1 or 2, or clear cell) or stages II–IV epithelial ovarian, primary peritoneal, or fallopian tube cancer [44]. All patients underwent first-line platinum-based chemotherapy and showed no signs of progression at the end of chemotherapy according to the Response Evaluation Criteria In Solid Tumors (RECIST) criteria [45] and/or the Gynecological Cancer Intergroup (GCIG) criteria in the case of CA125-based evaluation at the end of first-line treatment [46]. Overall, 835 patients were randomized 1:1 to maintenance erlotinib 150 mg orally daily for 2 years or observation. From the 835 patients registered in the trial, 527 patients(63 %) consented to optional translational research. Prospective bio-banking was performed, and formalin-fixed paraffin-embedded (FFPE) tissue sampled before and/or during first-line chemotherapy was requested from the participating centers for this translational study. The FFPE histological tissue was accepted independent of the site of biopsy—for example, surgical specimens from primary ovarian tumors, lymph nodes, or distant metastases. Patients gave written informed consent by signing the separate translational research informed consent form before any study-specific procedure. The translational research study and the informed consent forms were approved by the ethics committees of the institutions involved. The study was conducted in accordance with IHC good clinical practice guidelines and the Declaration of Helsinki, and was registered at ClinicalTrials.gov, number NCT00263822.

2.2 Molecular Tissue Biomarker Analyses

The translational analyses were performed at the Department of Oncology, Division of Gynecological Oncology, at the Catholic University Leuven (Leuven, Belgium), the Vesalius Research Center at the Flanders Institute for Biotechnology (VRC/VIB3) (Leuven, Belgium), and the University of Colorado Comprehensive Cancer Center (Aurora, CO, USA). Tumor tissues were sent as FFPE blocks, eppendorf tubes or glass slides. All tumor tissue samples were checked for quality, tissue integrity, and tumor content on a 5-μm hematoxylin and eosin (H&E) stained section. Tissue microarrays (TMAs) for the IHC analyses were constructed using a manual tissue puncher/arrayer (Beecher Instruments, Silver Spring, MD, USA). Tumor DNA was extracted using a phenol-chloroform method after macrodissection of the marked tumor region on the H&E slide. Biopsies with a low estimated tumor percentage on H&E and those failing to yield sufficient DNA after extraction were excluded (n=27).

2.2.1 Immunohistochemistry

Immunohistochemical analyses were performed on the constructed TMAs or individual slides using methods and assessment criteria described elsewhere [25]. The following primary antibodies were used for IHC: EGFR (Cell Signaling Technology, Inc., Beverly, MA, USA) diluted at 1:50, phosphorylated Akt (Ser473) at 1:50, phosphorylated p44/42 MAPK (Thr202/Tyr204; E10) monoclonal antibody (Cell Signaling Technology, Inc.) at 1:50, vimentin antibody V9 (Dako/Agilent Technologies, Carpinteria, CA, USA), and E-cadherin antibody H-108 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The percentage of total tumor cells within each staining intensity category [0 (no staining), 1+ (weak), 2+ (moderate), 3+ (strong)] was reported. A hybrid (H)-score was then generated based on the fraction of staining cells in each intensity category. The H-score was calculated by completing the formula (% cells of 0 intensity x 0)+(% of 1+ intensity x 1)+(% of 2+ intensity x 2)+(% of 3+ intensity x 3), producing a final H-score with a range of 0–300 [25]. For statistical analyses, the immunohistochemical results of EGFR, E-cadherin, pMAPK, and pAkt were analyzed in a binary fashion due to the skewed distribution of the results.

2.2.2 Fluorescence In Situ Hybridization (FISH) Analyses

We analyzed the *EGFR* gene copy number per cell using the LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen Probe (Abbott Molecular, Des Plaines, IL, USA), as previously described [19, 25]. Tumor specimens were classified into six FISH strata according to the frequency of cells with each *EGFR* gene copy number and referred to the chromosome 7 centromere, as follows: (1) disomy (three or four copies in <10 % of cells), (2) low trisomy (three copies in 10 % to <40 % of cells and four copies in <10 % of cells), (3) high trisomy (three copies in ≥40 % of cells and four copies in <10 % of cells), (4) low polysomy (four copies in 10 % to <40 % of cells), (5) high polysomy (four or more copies in ≥40 % of cells), and (6) gene amplification (presence of loose or tight *EGFR* gene clusters with ≥4 copies, *EGFR* gene-to-CEP 7 ratio≥2, or 15 copies of *EGFR* per cell in ≥10 % of cells). The high polysomy and gene amplification categories were considered to indicate a high *EGFR* copy number (*EGFR*-FISH positive), and the other categories were considered to indicate no significant increase in the *EGFR* copy number (*EGFR*-FISH negative), as previously described [34, 47].

Dual-target, dual-color *HER2* FISH assays were performed using the PathVysion HER-2 DNA probe kit (Abbott Molecular), which includes the LSI HER-2 SpectrumOrange and the CEP 17 SpectrumGreen probes. The reference slide (stained with H&E) was the adjacent section on which the dominant tumor foci were identified, and copy numbers of the *HER2* gene and chromosome 17 centromere probes were assessed and recorded independently in at least 50 non-overlapping nuclei with intact morphology. Analysis was performed blinded to the patients' clinical characteristics. Based on the mean number of copies of the *HER2* gene and chromosome 17 centromere per cell, patients were classified into three strata: negative for *HER2* amplification when the mean *HER2*/mean CEP17 ratio was <1.8, positive for *HER2* amplification when then mean *HER2*/mean CEP17 ratio was >2.2, and equivocal when the mean *HER2*/mean CEP17 ratio was between 1.8 and 2.2, in which case a second observer independently scored 50 tumor cells, and the final classification was assessed based on the results in all 100 cells.

2.2.3 Somatic Mutation Profiling

The COSMIC (Catalogue Of Somatic Mutations In Cancer) database [48] was queried for the most frequent mutations occurring in commonly mutated oncogenes related to the EGFR pathway, which resulted in coverage of >97 %, >94 %, >97 %, >79 %, >65 %, and >7 % for *KRAS*, *BRAF*, *NRAS*, *PIK3CA*, *EGFR*, and *PTEN*, respectively. DNA was aliquoted into 384-well plates and genotyped centrally at the Vesalius Research Center (Leuven, Belgium). iPLEX technology was used on a MassARRAY Compact Analyser (Sequenom Inc., San Diego, CA, USA), as described by De Roock et al. [31]. DNA of a sample was considered of sufficient quality when more than 75 % of mutations were reliably genotyped. A sample was considered wild-type for a given gene when the most frequently mutated sites in this gene did not show a mutation.

2.2.4 EGFR Sequencing

Targeted re-sequencing of *EGFR* was performed to identify potential somatic mutations that were not assessed in the Sequenom hotspot mutation panel. In particular, *EGFR* was sequenced using the TruSeq Custom Amplicon Kit on an

Illumina MiSeq sequencer (Illumina, Inc., San Diego, CA, USA). Custom oligo probes targeting *EGFR* exons were designed using DesignStudio (Illumina, Inc.). Amplicons were generated in an extension-ligation reaction across the region of

Table 2 Baseline patient characteristics: overall study population (n=835) and translational study population (mutation analysis n=318, FISH and immunohistochemical analysis n=218)

Parameter	Translational				Overall		P value	
	Mutation group (n=318)		FISH/IHC group (n=218)		(n=835)		Mutation vs overall	FISH/IHC vs overall
	No.	%	No.	%	No.	%		
Age (years)							0.300	0.532
Median	59.5		59.0		59.0			
Range	31–85		31–85		19–85			
WHO performance status							0.987	0.134
0	213	67	137	62.8	559	66.9		
1	105	33	81	37.2	276	33.1		
FIGO stage							0.561	0.128
I	25	7.9	21	9.6	57	6.8		
II	27	8.5	19	8.7	62	7.4		
III	212	66.7	144	66.1	563	67.4		
IV	54	17	33	15.1	152	18.2		
Unknown	0	0	1	0.5	1	0.1		
Histological grade							0.001	0.002
Grade 1	12	3.8	9	4.1	58	6.9		
Grade 2	58	18.2	38	17.4	152	18.2		
Grade 3	172	54.1	127	58.3	388	46.5		
Unknown	76	23.9	44	20.2	237	28.4		
Histological type							0.055	0.285
Serous	205	64.5	134	61.5	521	62.4		
Mucinous	7	2.2	6	2.8	14	1.7		
Clear cell	23	7.2	18	8.3	51	6.1		
Endometrioid	28	8.8	16	7.3	61	7.3		
Undifferentiated	8	2.5	7	3.2	21	2.5		
Other/Unknown	47	14.8	37	17	167	20		
Treatment arm							0.995	0.795
Erlotinib	160	50.3	108	49.5	420	50.3		
Observation	158	49.7	110	50.5	415	49.7		
First-line chemotherapy							0.612	0.693
Platinum alone	12	3.8	11	5	36	4.3		
Platinum doublet or triplet	306	96.2	207	95	799	95.7		
Response at end of first-line chemotherapy*							0.221	0.550
Complete remission	246	77.4	163	74.8	602	72.1		
Partial remission	65	20.4	49	22.5	204	24.4		
Stable disease	7	2.2	6	2.8	29	3.5		
Overall Survival (months)							0.272	0.588
Median	66.99		50.99		51[a]–59[b]			
Progression-free survival (months)							0.923	0.776
Median	12.8		13.04		12.4[b]-12.7[a]			

* according to the RECIST criteria and/or the GCIG criteria in the case of CA125-based evaluation at the end of first-line treatment [45, 46] [a] erlotinib arm[b] observation arm

FIGO Fédération Internationale de Gynécologie et d'Obstétrique, *WHO* World Health Organization

interest and amplified in a subsequent PCR, which also incorporates two unique sample-specific indexes, following the manufacturer's instructions. After pooling, the samples were sequenced on an Illumina MiSeq in a 2x150-bp paired-end sequencing run using a v2 flow cell. Data were mapped with the Burrows-Wheeler Aligner (bwa-0.5) [49] to the human reference genome hg19. The Genome Analysis Toolkit (GATK version 1.6; Broad Institute) was further used to process the data, and variants and indels were called by the Unified Genotyper and Dindel, respectively [50, 51]. The mutations were further filtered [52] and manually curated in IGV. *EGFR* re-sequencing was successful if the average coverage per sample was >100x.

2.3 Statistical Analyses

All statistical analyses for the translational study of the EORTC-GCG 55041 trial were performed centrally at the headquarters of the EORTC in Brussels (Belgium). The

Table 3 Correlation of biomarker results with overall survival (OS) and progression-free survival (PFS)

Biomarker	*n*	OS			PFS		
		Mos.	HR (95 % CI)	*P*	Mos.	HR (95 % CI)	*P*
KRAS							
Wild-type	309	NR			12.5		
Mutation	9	67.0	1.08 (0.43–2.69)	0.876	14.1	0.90 (0.42–1.91)	0.784
PIK3CA							
Wild-type	306	67.0			12.4		
Mutation	12	NR	0.53 (0.17–1.65)	0.262	NR	0.36 (0.15–0.86)	**0.017**
EGFR							
Wild-type	315	NR			12.9		
Mutation	3	37.9	1.80 (0.53–6.15)	0.345	4.01	2.09 (0.67–6.54)	0.195
KRAS, NRAS, BRAF, PIK3CA or EGFR							
Wild-type	294	NR			12.3		
Mutation	24	67.0	0.75 (0.38–1.49)	0.413	33.1	0.57 (0.33–0.99)	**0.042**
EGFR IHC							
Negative	125	51.0			11.7		
Positive	93	46.1	1.23 (0.85–1.78)	0.273	13.0	1.03 (0.76–1.39)	0.835
EGFR FISH							
Negative	114	67.0			16.1		
Positive	66	46.1	1.56 (1.01–2.40)	**0.044**	9.6	1.57 (1.11–2.22)	**0.010**
pAkt-IHC							
Negative	108	49.1			11.6		
Positive	27	53.7	0.89 (0.48–1.63)	0.696	13.8	0.65 (0.39–1.09)	0.100
pMAPK-IHC							
Negative	70	67.0			15.7		
Positive	58	38.9	1.81 (1.11–2.97)	**0.016**	8.1	1.42 (0.96–2.10)	0.077
E-cadherin-IHC							
Negative	64	67.0			10.5		
Positive	71	51.0	0.99 (0.61–1.62)	0.970	13.1	0.87 (0.59–1.28)	0.491
Vimentin-IHC							
Negative	30	NR			13.8		
Intermediate	58	49.0	1.61 (0.78–3.30)		13.1	0.88 (0.53–1.45)	
Positive	40	39.6	2.07 (0.99–4.34)	0.139	9.1	1.18 (0.70–1.98)	0.430
HER2-FISH							
Negative	30	49.1			22.1		
Equivocal	23	51.0	1.07 (0.48–2.35)		8.8	2.13 (1.13–4.01)	
Positive	53	67.0	0.97 (0.49–1.89)	0.965	13.7	1.51 (0.87–2.63)	0.061

CI confidence interval, *FISH* fluorescence in situ hybridization, *HR* hazard ratio, *IHC* immunohistochemistry, *Mos*. months, *NR* not reached, *OS* overall survival, *PFS* progression-free survival. Bold entries indicate statistically significant p values (P <0.05)

sample size of the trial (n=835) was based on the primary endpoint, progression-free survival (PFS), to detect an increase of 25 % in the median PFS from 15 to 18.75 months [44]. Secondary endpoints were overall survival (OS), toxicity, occurrence of rash, and quality of life. OS and PFS were defined as the time from randomization to the date of the event (death and death or progression, respectively). Time-to-event analyses were investigated via the Kaplan-Meier method with hazard ratios (HR) obtained through Cox regression and compared via a nonparametric log-rank test stratified for treatment (where possible). Predictiveness for treatment effect was assessed via Cox regression using an interaction test with the allocated protocol treatment (erlotinib versus observation). All tests were nonparametric two-sided tests, evaluated at the 5 % significance level. No correction for multiplicity was made, and the 5 % level was used as a screening measure rather than a formal hypothesis test.

3 Results

3.1 Patient Characteristics

Tumor tissues were available for 358 of the 527 patients (68 %) who consented to optional translational research. Mutation data were available for 318 patients and FISH and IHC data for 218 patients. The main reason for the different numbers was due to the fact that DNA extraction was possible in most samples, where IHC or FISH analysis was not achievable due to technical reasons. Detailed patient characteristics are summarized in Table 2. With regard to important baseline parameters (e.g., age, stage, histological type and grade) no significant imbalances between the overall study population and the translational study population were apparent, except for histological grade (with more high-grade tumors and fewer patients with unknown histological grade in the translational study population).

3.2 Frequency of Alterations in Molecular EGFR Pathway Biomarkers and Clinical Outcome

Table 3 summarizes the correlation between biomarker results and both PFS and OS. The distribution of selected baseline patient characteristics between the molecular marker results are summarized in Online Resource 1.

3.2.1 EGFR Protein Expression

EGFR protein expression was evaluated by immunohistochemistry in 218 patients. Of these, 42.7 % had EGFR membranous staining (EGFR IHC-positive) (Fig. 1). EGFR protein expression had no impact on OS (HR: 1.23, $P = 0.273$) or PFS (HR: 1.03, $P = 0.835$).

3.2.2 EGFR Gene Copy Number

We assessed *EGFR* gene copy number by FISH in 180 patients. Patients with high polysomy and gene amplification categories were combined and designated EGFR FISH-positive, and all other categories were categorized as *EGFR* FISH-negative, as previously described [19]. Representative images of these two classes are shown in Fig. 2. FISH-positive patients represented 36.7 % of the total group, with 63.3 % FISH-negative. Next, we correlated *EGFR* FISH categories with clinical outcome (Fig. 3). Compared to FISH-negative patients, *EGFR* FISH-positive patients had

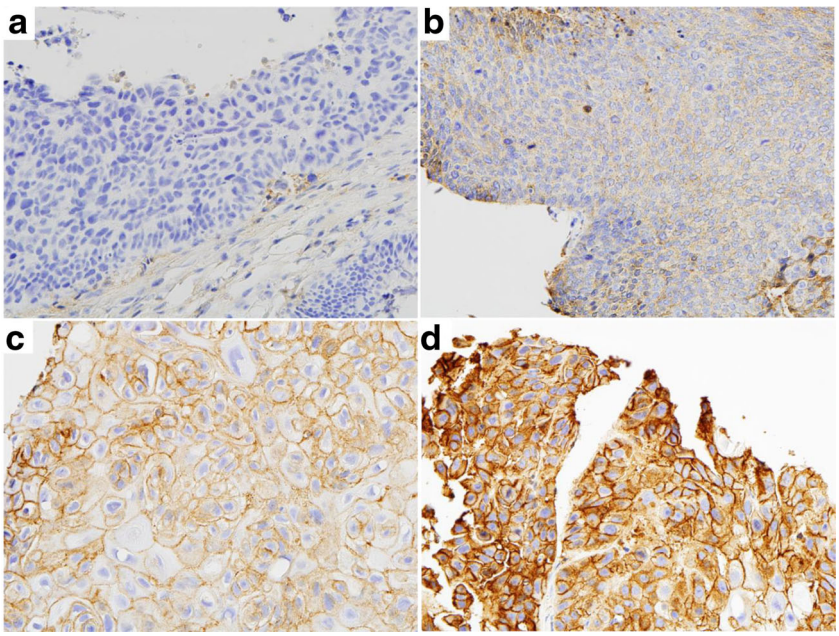


Fig. 1 EGFR immunohistochemical staining. A hybrid (H)-score was generated based on the fraction of staining cells in each intensity category [0 (no staining), 1+ (weak), 2+ (moderate), 3+ (strong)]. The H-score was calculated by completing the formula (% cells of 0 intensity x 0)+(% of 1+ intensity x 1)+(% of 2+ intensity x 2)+(% of 3+ intensity x 3), with the overall score ranging from 0 to 300. Panels illustrate specimens graded with score 0 (**a**), score 80 (low expression, **b**), score 170 (moderate expression, **c**) and score 280 (high expression, **d**) (image magnification 40x)

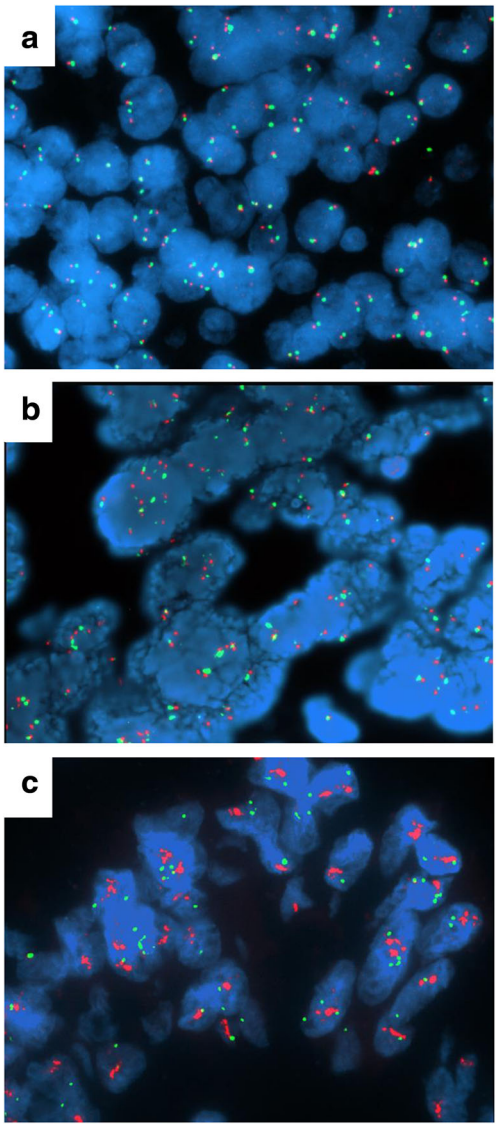


Fig. 2 *EGFR* determined by fluorescence in situ hybridization. FISH was performed with the EGFR (red)/CEP7 (green) probe (Abbott Molecular, Des Plaines, IL, USA). Panels illustrate specimens representing low gain in gene copy number per cell (*EGFR* FISH-negative) (**a**), high (high polysomy=**b**; gene amplification=**c**) gain in gene copy number per cell (EGFR FISH-positive)

statistically significantly shorter OS (median 46.1 versus 67.0 months, HR=1.56 [95 % CI 1.01-2.40], P=0.044) and shorter PFS (median 9.6 versus 16.1 months, HR=1.57 [95 % CI1.11–2.22], P=0.010). However, *EGFR* gene copy number could not predict responsiveness to erlotinib (Fig. 4).

3.2.3 pAkt and pMAPK Expression by IHC

Immunohistochemical evaluation of pAkt and pMAPK was successful in 135 and 128 patients, respectively, with 27 cases (20 %) for pAkt and 58 cases (45.3 %) for pMAPK classified as positive. Positive pMAPK expression was seen mainly in serous tumors. While no effect of pAkt IHC positivity was seen on OS (HR: 0.89, P=0.696) or PFS (HR: 0.65, P=0.100), pMAPK IHC positivity was significantly associated with worse OS (HR: 1.81, P=0.016) (Fig. 3). pMAPK did not seem to have predictive value (Fig. 4).

3.2.4 Epithelial-Mesenchymal Transition (EMT) Status by IHC

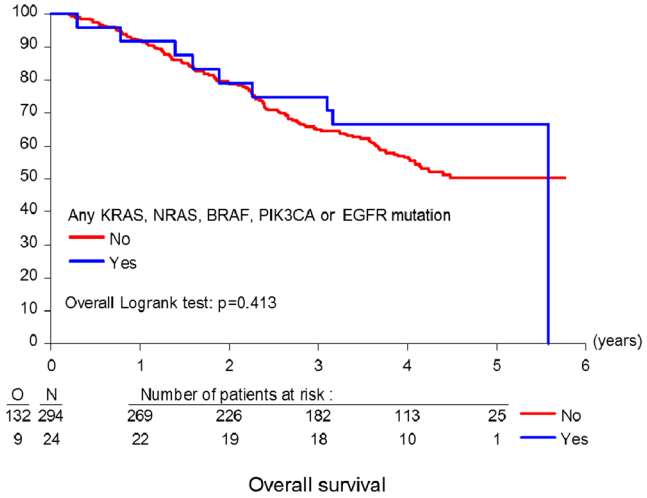
Changes in EMT status including the expression of E-cadherin as epithelial marker and vimentin as mesenchymal marker were successfully evaluated by IHC in 135 and 128 patients, respectively. The protein expression of E-cadherin was considered high in 52.6 % of patients, while vimentin was categorized as low (≤30), intermediate (30–130), or high (>130). High protein expression of vimentin was observed in 31.3 %. Neither E-cadherin nor vimentin IHC correlated with outcome or were predictive (Table 3).

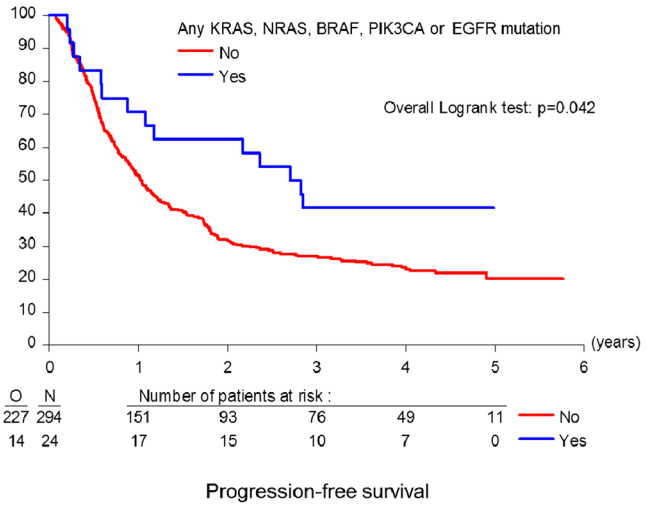
3.2.5 HER2 Gene Copy Number Status by FISH

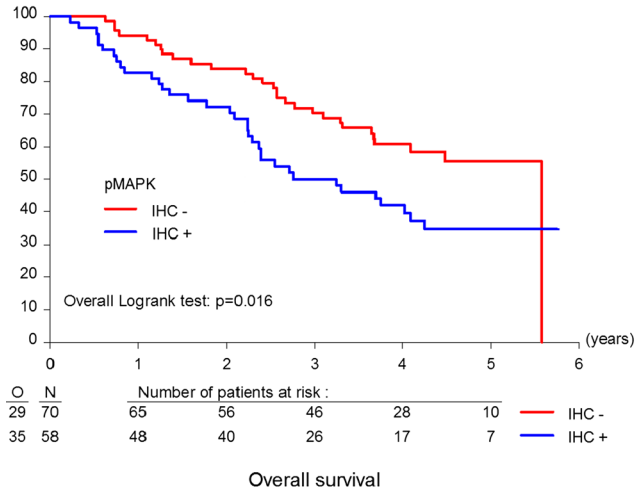
HER2 FISH was assessable in 106 patients with available tumor tissue and classified as positive (>2.2) in 50 % of cases. No significant correlation between *HER2* copy number status and outcome was observed (Table 3).

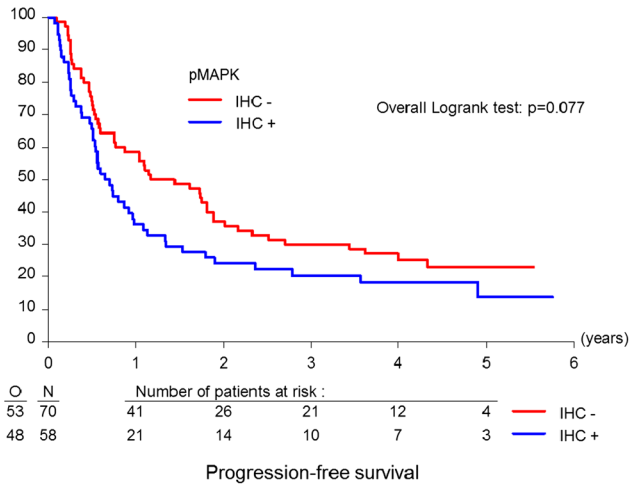
3.2.6 Mutation Analysis

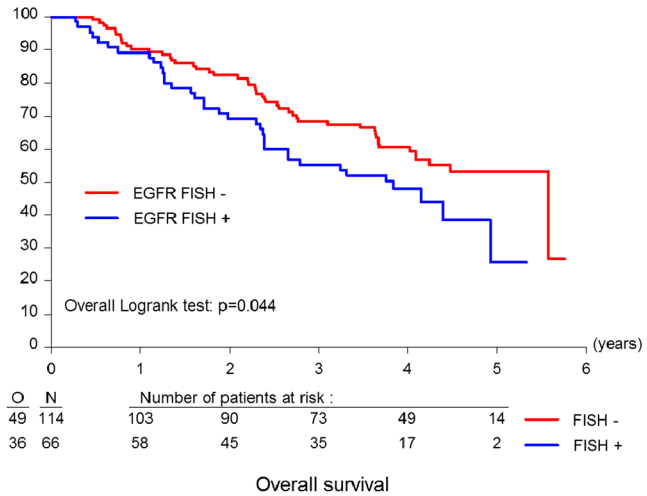
Hotspot mutation profiling of 20, 2, 20, 33, 15, and 8 somatic mutations in *KRAS*, *BRAF*, *NRAS*, *PIK3CA*, *EGFR*, and *PTEN*, respectively, using Sequenom MassARRAY was technically successful for 318 cases (Online Resource 2). Overall, only 28 mutations were observed among 24 samples (7.5 %). Of the genes analyzed, *PIK3CA* and *KRAS* were the most frequently mutated. *PIK3CA* mutations were present in 3.8 % (12/318) of the samples, most of which were located in exon 9 (4/12; 33 %) or exon 20 (4/12; 33 %). *KRAS* mutations were detected in nine samples (2.8 %), nearly all within codon 12 (8/9, 88.9 %). *EGFR* mutations occurred in only three patients. *EGFR* re-sequencing was successful in 59 of 64 samples. The average coverage of *EGFR* exons for these samples was 590x. No additional somatic mutations were detected in any of the *EGFR* exons. Mutations in *PTEN* were not detected. Mutations occurred less frequently in serous cancers (7/206 [3 %] in serous versus 17/112 [15 %] in non-serous cancers; see Online Resource 1). The limited frequency of mutated samples in this analysis prevents a meaningful evaluation of clinical outcomes in relation to individual mutations. As such, patients with at least one mutation in the *EGFR* signaling cascades were pooled. Patients with at least one mutation in *KRAS*, *NRAS*, *BRAF*, *PIK3CA*, or *EGFR* had longer PFS (33.1 versus 12.3 months, mean difference=20.8 months, P=











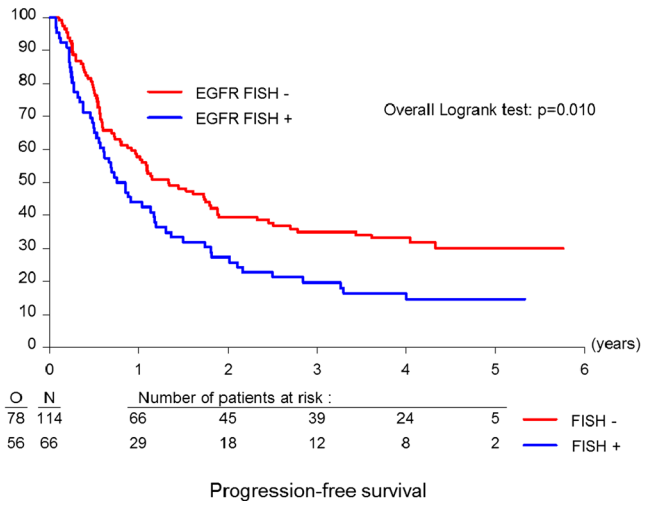


Fig. 3 Kaplan-Meier curves for overall survival and progression-free survival. Data were analyzed according to presence of mutations (top), pMAPK immunohistochemistry (middle), and EGFR gene copy number (bottom)

0.042) compared to those with wild-type tumors (Fig. 3), but among the former, there was no significant benefit of erlotinib over observation (Fig. 4). No significant difference in OS was seen when all mutations were pooled.

4 Discussion

Most data that have added to our understanding of molecular responsiveness to anti-EGFR agents have been derived from

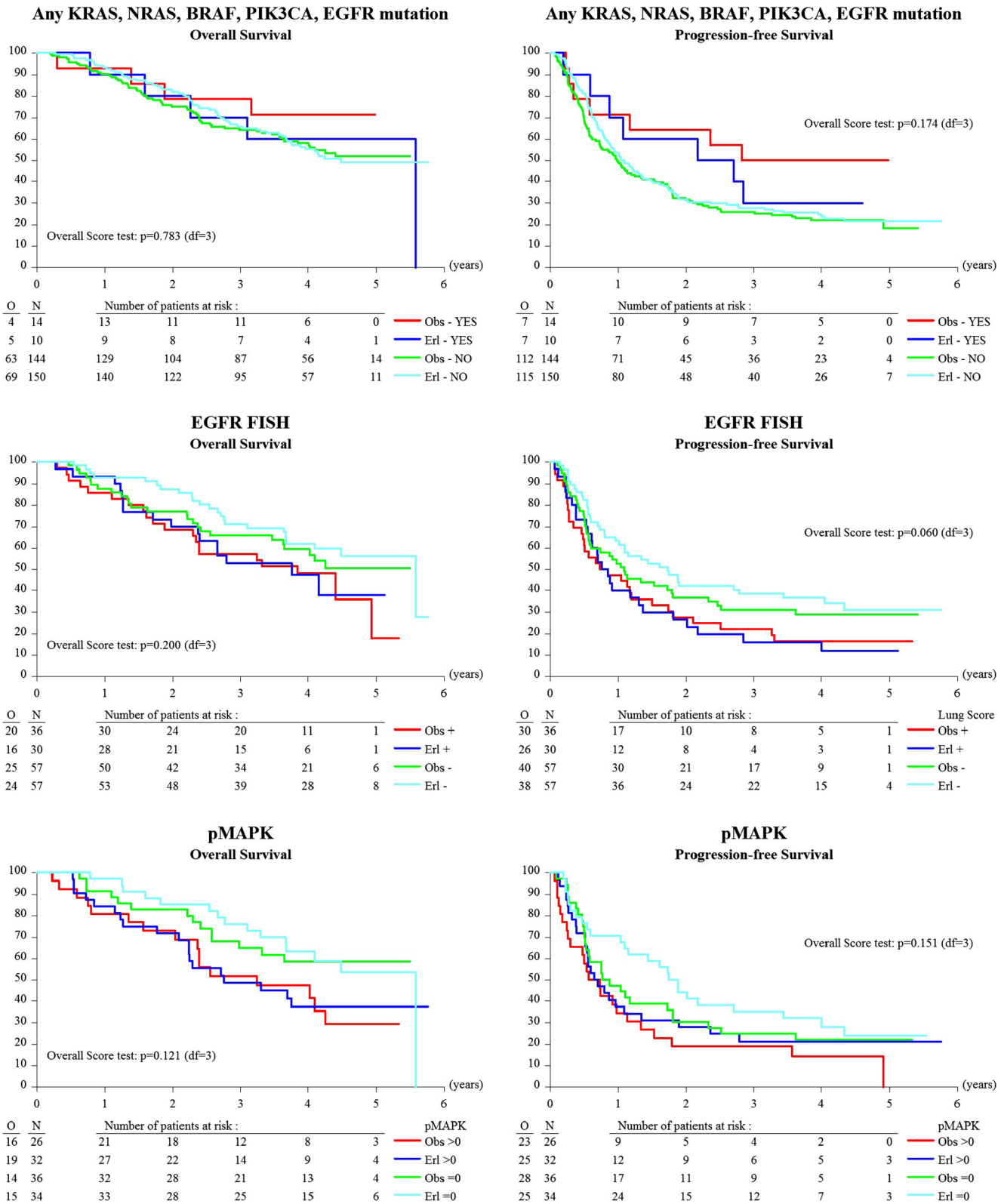


Fig. 4 Kaplan-Meier curves for overall survival and progression-free survival according to treatment allocation. Data were analyzed according to presence of mutations (top), pMAPK immunohistochemistry (middle), and EGFR gene copy number (bottom)

well-conducted trials in mCRC and NSCLC. To date, only limited data on EGFR pathway biomarkers are available for epithelial ovarian cancer and, even more specifically, in patients being treated with anti-EGFR therapies. The EORTC-GCG 55041 trial is the first randomized phase III trial investigating the use of maintenance erlotinib in patients with ovarian, peritoneal or fallopian tube cancer. In this project, we investigated the status of EGFR and related pathways using immunohistochemistry, FISH, hotspot mutation analysis, and DNA sequencing to determine the frequency of these alterations in patients with ovarian cancer and to correlate these biomarker data with outcome and with the efficacy of erlotinib.

In this study, EGFR overexpression was found with IHC in 39.4 % of patients, but we could not validate EGFR expression as a poor prognostic marker. The prognostic role of EGFR expression in EOC remains controversial. While some studies associate EGFR overexpression with poor clinical outcome [3, 5], others have shown no effect [6, 7]. However, we found a statistically significant correlation with PFS and OS for *EGFR* copy number status. Patients who were *EGFR* FISH-positive had worse OS (46.1 months) than those who were *EGFR* FISH-negative (67.0 months) (HR: 1.56; 95 % CI 1.01–2.40; P=0.044). The median PFS was 9.6 months he *EGFR* FISH-positive patients and 16.1 months for *EGFR* FISH-negative patients (HR: 1.57; 95 % CI 1.11–2.22; P=0.010). However, *EGFR* amplification was not predictive of erlotinib responsiveness, suggesting that *EGFR* copy number status may be a prognostic rather than predictive factor for erlotinib. Results reported by Lassus et al. also support the assumption that *EGFR* copy number serves as a prognostic biomarker in EOC, showing that an increased *EGFR* copy number was associated with shorter OS and PFS [53].

In contrast to mCRC and NSCLC, *EGFR* mutation did not predict responsiveness to erlotinib treatment, nor did gain-of-function mutations in the *EGFR* signaling cascades (*KRAS*, *BRAF*, *NRAS*, and *PIK3CA*). The limited frequency of mutated samples, however, prevents meaningful evaluation of clinical outcomes in relation to these mutations. However, when pooling all mutations together, patients with at least one mutation in either *KRAS*, *NRAS*, *BRAF*, *PIK3CA* or *EGFR* had a longer PFS (33.1 months) compared to those with wild-type tumors (12.3 months) (HR: 0.57; 95 % CI 0.33-0.99; P=0.042).

With the exception of pMAPK expression, which was associated with shorter overall survival, none of the investigated biomarkers demonstrated prognostic significance or were predictive of erlotinib efficacy in our translational study population. Some limitations to the study, however, must be taken into consideration. Although there was a compelling body of basic and preclinical evidence for launching this study, it failed to show a benefit of erlotinib over standard management (observation) [44], making it very difficult to evaluate predictive biomarkers. In addition, 26 % of the patients stopped erlotinib due to side effects. Moreover, obtaining a full analysis data set with complete results for all tissue samples submitted to molecular analysis remains a challenge. The different techniques often require fresh tissue or a sufficient amount of adequate tissue or tumor cells. Thus, tissue quality and technical performance may also significantly affect biostatistical results. Finally, EOC represents a genetically complex disease, rendering simple translation of the biomarker data difficult.

Although the EGFR pathway appears to play an important role in ovarian cancer, particularly in tumor development, tumor cell survival, and metastasis, it is not yet clear how this pathway can be exploited to yield a therapeutic benefit. In the future, rather than using an unselected patient population, it will be important to select patients based only on molecular characteristics for randomized studies investigating EGFR-targeted treatments. In the event that findings are similar to those in breast cancer for HER2 and trastuzumab or in NSCLC for EGFR and gefitinib, where increased copy number is a poor prognostic feature but a good predictor of response, *EGFR* copy number status may represent a criterion for selecting EOC patients for those clinical trials.

In conclusion, in this translational study on the EORTC-GCG 55041 trial, the presence of *EGFR* mutations or gain-of-function mutations in the *EGFR* signaling cascades (*KRAS*, *BRAF*, *NRAS*, and *PIK3CA*), increased *EGFR* copy number, and positive EGFR protein overexpression were not predictive of erlotinib efficacy. However, increased *EGFR* copy number seems to have a prognostic role. Therefore, *EGFR* FISH analysis may be interesting for further investigation as a criterion for selecting patients for prospective studies on erlotinib or other anti-EGFR therapies in EOC.

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Conflict of Interest Marileila Varella-Garcia is co-inventor on a patent held by the University of Colorado to use EGFR copy number as biomarker for selection of lung cancer patients for targeted therapy. Evelyn Despierre, Ignace Vergote, Ryan Anderson, Corneel Coens, Dionyssios Katsaros, Fred R. Hirsch, Bram Boeckx, Annamaria Ferrero, Isabelle Ray-Coquard, Els MJJ Berns, Antonio Casado, Diether Lambrechts, and Antonio Jimeno declare no conflict of interest.

References

1. Ciardiello F, Tortora G (2008) EGFR antagonists in cancer treatment. N Engl J Med 358(11):1160–74. doi:10.1056/NEJMra0707704
2. Yarden Y, Sliwkowski MX (2001) Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2(2):127–37
3. Skirnisdóttir I, Sorbe B, Seidal T (2001) The growth factor receptors HER-2/neu and EGFR, their relationship, and their effects on the prognosis in early stage (FIGO I-II) epithelial ovarian carcinoma. Int J Gynecol Cancer 11(2):119–29
4. Fischer-Colbrie J, Witt A, Heinzl H, Speiser P, Czerwenka K, Sevelda P, Zeillinger R (1997) EGFR and steroid receptors in ovarian carcinoma: comparison with prognostic parameters and outcome of patients. Anticancer Res 17(1B):613–9
5. Berchuck A, Rodriguez GC, Kamel A, Dodge RK, Soper JT, Clarke-Pearson DL, Bast RC Jr (1991) Epidermal growth factor receptor expression in normal ovarian epithelium and ovarian cancer. I. Correlation of receptor expression with prognostic factors in patients with ovarian cancer. Am J Obstet Gynecol 164(2):669–74
6. Baekelandt M, Kristensen GB, Tropé CG, Nesland JM, Holm R (1999) Epidermal growth factor receptor expression has no independent prognostic significance in advanced ovarian cancer. Anticancer Res 19(5C):4469–74
7. Elie C, Geay JF, Morcos M, Le Tourneau A, Girre V, Broët P, Marmey B, Chauvenet L, Audouin J, Pujade-Lauraine E, Camilleri-Broët S, GINECO Group (2004) Lack of relationship between EGFR-1 immunohistochemical expression and prognosis in a multicentre clinical trial of 93 patients with advanced primary ovarian epithelial cancer (GINECO group). Br J Cancer 91(3):470–5
8. Gordon AN, Finkler N, Edwards RP, Garcia AA, Crozier M, Irwin DH, Barrett E (2005) Efficacy and safety of erlotinib HCl, an epidermal growth factor receptor (HER1/EGFR) tyrosine kinase inhibitor, in patients with advanced ovarian carcinoma: results from a phase II multicenter study. Int J Gynecol Cancer 15(5):785–92
9. Blank SV, Christos P, Curtin JP, Goldman N, Runowicz CD, Sparano JA, Liebes L, Chen HX, Muggia FM (2010) Erlotinib added to carboplatin and paclitaxel as first-line treatment of ovarian cancer: a phase II study based on surgical reassessment. Gynecol Oncol 119(3):451–6. doi:10.1016/j.ygyno.2010.08.008
10. Hirte H, Oza A, Swenerton K, Ellard SL, Grimshaw R, Fisher B, Tsao M, Seymour L (2010) A phase II study of erlotinib (OSI-774) given in combination with carboplatin in patients with recurrent epithelial ovarian cancer (NCIC CTG IND.149). Gynecol Oncol 118(3):308–12. doi:10.1016/j.ygyno.2010.05.005

11. Schilder RJ, Sill MW, Chen X, Darcy KM, Decesare SL, Lewandowski G, Lee RB, Arciero CA, Wu H, Godwin AK (2005) Phase II study of gefitinib in patients with relapsed or persistent ovarian or primary peritoneal carcinoma and evaluation of epidermal growth factor receptor mutations and immunohistochemical expression: a Gynecologic Oncology Group Study. Clin Cancer Res 11(15):5539–48
12. Posadas EM, Liel MS, Kwitkowski V, Minasian L, Godwin AK, Hussain MM, Espina V, Wood BJ, Steinberg SM, Kohn EC (2007) A phase II and pharmacodynamic study of gefitinib in patients with refractory or recurrent epithelial ovarian cancer. Cancer 109(7):1323–30
13. Wagner U, du Bois A, Pfisterer J, Huober J, Loibl S, Lück HJ, Sehouli J, Gropp M, Stähle A, Schmalfeldt B, Meier W, Jackisch C, AGO Ovarian Cancer Study Group (2007) Gefitinib in combination with tamoxifen in patients with ovarian cancer refractory or resistant to platinum-taxane based therapy–a phase II trial of the AGO Ovarian Cancer Study Group (AGO-OVAR 2.6). Gynecol Oncol 105(1):132–7
14. Pautier P, Joly F, Kerbrat P, Bougnoux P, Fumoleau P, Petit T, Rixe O, Ringeisen F, Carrasco AT, Lhommé C (2010) Phase II study of gefitinib in combination with paclitaxel (P) and carboplatin (C) as second-line therapy for ovarian, tubal or peritoneal adenocarcinoma (1839IL/0074). Gynecol Oncol 116(2):157–62. doi:10.1016/j.ygyno.2009.10.076
15. Schilder RJ, Pathak HB, Lokshin AE, Holloway RW, Alvarez RD, Aghajanian C, Min H, Devarajan K, Ross E, Drescher CW, Godwin AK (2009) Phase II trial of single agent cetuximab in patients with persistent or recurrent epithelial ovarian or primary peritoneal carcinoma with the potential for dose escalation to rash. Gynecol Oncol 113(1):21–7. doi:10.1016/j.ygyno.2008.12.003
16. Secord AA, Blessing JA, Armstrong DK, Rodgers WH, Miner Z, Barnes MN, Lewandowski G, Mannel RS, Gynecologic Oncology Group (2008) Phase II trial of cetuximab and carboplatin in relapsed platinum-sensitive ovarian cancer and evaluation of epidermal growth factor receptor expression: a Gynecologic Oncology Group study. Gynecol Oncol 108(3):493–9. doi:10.1016/j.ygyno.2007.11.029
17. Cannistra SA (2004) Cancer of the ovary. N Engl J Med 351(24): 2519–29
18. Bristow RE, Tomacruz RS, Armstrong DK, Trimble EL, Montz FJ (2002) Survival effect of maximal cytoreductive surgery for advanced ovarian carcinoma during the platinum era: a meta-analysis. J Clin Oncol 20(5):1248–59
19. Cappuzzo F, Hirsch FR, Rossi E, Bartolini S, Ceresoli GL, Bemis L, Haney J, Witta S, Danenberg K, Domenichini I, Ludovini V, Magrini E, Gregorc V, Doglioni C, Sidoni A, Tonato M, Franklin WA, Crino L, Bunn PA Jr, Varella-Garcia M (2005) Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. J Natl Cancer Inst 97(9):643–55
20. Hirsch FR, Varella-Garcia M, McCoy J, West H, Xavier AC, Gumerlock P, Bunn PA Jr, Franklin WA, Crowley J, Gandara DR, Southwest Oncology Group (2005) Increased epidermal growth factor receptor gene copy number detected by fluorescence in situ hybridization associates with increased sensitivity to gefitinib in patients with bronchioloalveolar carcinoma subtypes: a Southwest Oncology Group Study. J Clin Oncol 23(28):6838–45
21. Chung KY, Shia J, Kemeny NE, Shah M, Schwartz GK, Tse A, Hamilton A, Pan D, Schrag D, Schwartz L, Klimstra DS, Fridman D, Kelsen DP, Saltz LB (2005) Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. J Clin Oncol 23(9):1803–10
22. Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 304(5676):1497–500
23. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 350(21):2129–39
24. Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, Singh B, Heelan R, Rusch V, Fulton L, Mardis E, Kupfer D, Wilson R, Kris M, Varmus H (2004) EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A 101(36):13306–11
25. Hirsch FR, Varella-Garcia M, Bunn PA Jr, Di Maria MV, Veve R, Bremmes RM, Barón AE, Zeng C, Franklin WA (2003) Epidermal growth factor receptor in non-small-cell lung carcinomas: correlation between gene copy number and protein expression and impact on prognosis. J Clin Oncol 21(20):3798–807
26. Moroni M, Veronese S, Benvenuti S, Marrapese G, Sartore-Bianchi A, Di Nicolantonio F, Gambacorta M, Siena S, Bardelli A (2005) Gene copy number for epidermal growth factor receptor (EGFR) and clinical response to antiEGFR treatment in colorectal cancer: a cohort study. Lancet Oncol 6(5):279–86
27. Cappuzzo F, Finocchiaro G, Rossi E, Jänne PA, Carnaghi C, Calandri C, Bencardino K, Ligorio C, Ciardiello F, Pressiani T, Destro A, Roncalli M, Crino L, Franklin WA, Santoro A, Varella-Garcia M (2008) EGFR FISH assay predicts for response to cetuximab in chemotherapy refractory colorectal cancer patients. Ann Oncol 19(4):717–23
28. Sartore-Bianchi A, Moroni M, Veronese S, Carnaghi C, Bajetta E, Luppi G, Sobrero A, Barone C, Cascinu S, Colucci G, Cortesi E, Nichelatti M, Gambacorta M, Siena S (2007) Epidermal growth factor receptor gene copy number and clinical outcome of metastatic colorectal cancer treated with panitumumab. J Clin Oncol 25(22):3238–45
29. Di Nicolantonio F, Martini M, Molinari F, Sartore-Bianchi A, Arena S, Saletti P, De Dosso S, Mazzucchelli L, Frattini M, Siena S, Bardelli A (2008) Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. J Clin Oncol 26(35):5705–12. doi:10.1200/JCO.2008.18.0786
30. Frattini M, Saletti P, Romagnani E, Martin V, Molinari F, Ghisletta M, Camponovo A, Etienne LL, Cavalli F, Mazzucchelli L (2007) PTEN loss of expression predicts cetuximab efficacy in metastatic colorectal cancer patients. Br J Cancer 97(8):1139–45
31. De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, Kalogeras KT, Kotoula V, Papamichael D, Laurent-Puig P, Penault-Llorca F, Rougier P, Vincenzi B, Santini D, Tonini G, Cappuzzo F, Frattini M, Molinari F, Saletti P, De Dosso S, Martini M, Bardelli A, Siena S, Sartore-Bianchi A, Tabernero J, Macarulla T, Di Fiore F, Gangloff AO, Ciardiello F, Pfeiffer P, Qvortrup C, Hansen TP, Van Cutsem E, Piessevaux H, Lambrechts D, Delorenzi M, Tejpar S (2010) Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol 11(8):753–62
32. Linardou H, Dahabreh IJ, Kanaloupiti D, Siannis F, Bafaloukos D, Kosmidis P, Papadimitriou CA, Murray S (2008) Assessment of somatic k-RAS mutations as a mechanism associated with resistance to EGFR-targeted agents: a systematic review and meta-analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer. Lancet Oncol 9(10):962–72. doi:10.1016/S1470-2045(08)70206-7
33. Van Cutsem E, Köhne CH, Láng I, Folprecht G, Nowacki MP, Cascinu S, Shchepotin I, Maurel J, Cunningham D, Tejpar S, Schlichting M, Zubel A, Celik I, Rougier P, Ciardiello F (2011) Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-

line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. J Clin Oncol 29(15):2011–9. doi:10.1200/JCO.2010.33.5091

34. Massarelli E, Varella-Garcia M, Tang X, Xavier AC, Ozburn NC, Liu DD, Bekele BN, Herbst RS, Wistuba II (2007) KRAS mutation is an important predictor of resistance to therapy with epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer. Clin Cancer Res 13(10):2890–6
35. Godin-Heymann N, Ulkus L, Brannigan BW, McDermott U, Lamb J, Maheswaran S, Settleman J, Haber DA (2008) The T790M "gatekeeper" mutation in EGFR mediates resistance to low concentrations of an irreversible EGFR inhibitor. Mol Cancer Ther 7(4):874–9. doi:10.1158/1535-7163.MCT-07-2387
36. Cappuzzo F, Ligorio C, Toschi L, Rossi E, Trisolini R, Paioli D, Magrini E, Finocchiaro G, Bartolini S, Cancellieri A, Hirsch FR, Crino L, Varella-Garcia M (2007) EGFR and HER2 gene copy number and response to first-line chemotherapy in patients with advanced non-small cell lung cancer (NSCLC). J Thorac Oncol 2(5):423–9
37. Daniele L, Macrì L, Schena M, Dongiovanni D, Bonello L, Armando E, Ciuffreda L, Bertetto O, Bussolati G, Sapino A (2007) Predicting gefitinib responsiveness in lung cancer by fluorescence in situ hybridization/chromogenic in situ hybridization analysis of EGFR and HER2 in biopsy and cytology specimens. Mol Cancer Ther 6(4):1223–9
38. Cappuzzo F, Varella-Garcia M, Shigematsu H, Domenichini I, Bartolini S, Ceresoli GL, Rossi E, Ludovini V, Gregorc V, Toschi L, Franklin WA, Crino L, Gazdar AF, Bunn PA Jr, Hirsch FR (2005) Increased HER2 gene copy number is associated with response to gefitinib therapy in epidermal growth factor receptor-positive non-small-cell lung cancer patients. J Clin Oncol 23(22):5007–18
39. Hirsch FR, Varella-Garcia M, Cappuzzo F (2009) Predictive value of EGFR and HER2 overexpression in advanced non-small-cell lung cancer. Oncogene 28(Suppl 1):S32–7. doi:10.1038/onc.2009.199
40. Yonesaka K, Zejnullahu K, Okamoto I, Satoh T, Cappuzzo F, Souglakos J, Ercan D, Rogers A, Roncalli M, Takeda M, Fujisaka Y, Philips J, Shimizu T, Maenishi O, Cho Y, Sun J, Destro A, Taira K, Takeda K, Okabe T, Swanson J, Itoh H, Takada M, Lifshits E, Okuno K, Engelman JA, Shivdasani RA, Nishio K, Fukuoka M, Varella-Garcia M, Nakagawa K, Jänne PA (2011) Activation of ERBB2 signaling causes resistance to the EGFR-directed therapeutic antibody cetuximab. Sci Transl Med 3(99):99ra86. doi:10.1126/scitranslmed.3002442
41. Martin V, Landi L, Molinari F, Fountzilas G, Geva R, Riva A, Saletti P, De Dosso S, Spitale A, Tejpar S, Kalogeras KT, Mazzucchelli L, Frattini M, Cappuzzo F (2013) HER2 gene copy number status may influence clinical efficacy to anti-EGFR monoclonal antibodies in metastatic colorectal cancer patients. Br J Cancer 108(3):668–75. doi:10.1038/bjc.2013.4
42. Frederick BA, Helfrich BA, Coldren CD, Zheng D, Chan D, Bunn PA Jr, Raben D (2007) Epithelial to mesenchymal transition predicts gefitinib resistance in cell lines of head and neck squamous cell carcinoma and non-small cell lung carcinoma. Mol Cancer Ther 6(6):1683–91
43. Uramoto H, Iwata T, Onitsuka T, Shimokawa H, Hanagiri T, Oyama T (2010) Epithelial-mesenchymal transition in EGFR-TKI acquired resistant lung adenocarcinoma. Anticancer Res 30(7):2513–7
44. Vergote IB, Jimeno A, Joly F, Katsaros D, Coens C, Despierre E, Marth C, Hall M, Steer CB, Colombo N, Lesoin A, Casado A, Reinthaller A, Green J, Buck M, Ray-Coquard I, Ferrero A, Favier L, Reed NS, Curé H, Pujade-Lauraine E (2014) Randomized phase III study of erlotinib versus observation in patients with no evidence of disease progression after first-line platin-based chemotherapy for ovarian carcinoma: a European Organisation for Research and Treatment of Cancer-Gynaecological Cancer Group, and Gynecologic Cancer Intergroup study. J Clin Oncol 32(4):320–6. doi:10.1200/JCO.2013.50.5669
45. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG (2000) New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 92(3):205–16
46. Vergote I, Rustin GJ, Eisenhauer EA, Kristensen GB, Pujade-Lauraine E, Parmar MK, Friedlander M, Jakobsen A, Vermorken JB (2000) Re: new guidelines to evaluate the response to treatment in solid tumors [ovarian cancer]. Gynecologic Cancer Intergroup. J Natl Cancer Inst 92(18):1534–5
47. Varella-Garcia M (2006) Stratification of non-small cell lung cancer patients for therapy with epidermal growth factor receptor inhibitors: the EGFR fluorescence in situ hybridization assay. Diagn Pathol 1:19
48. Forbes SA, Bindal N, Bamford S, Cole C, Kok CY, Beare D, Jia M, Shepherd R, Leung K, Menzies A, Teague JW, Campbell PJ, Stratton MR, Futreal PA (2011) COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. Nucleic Acids Res 39(Database issue):D945–50. doi:10.1093/nar/gkq929
49. Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25(14):1754–60. doi:10.1093/bioinformatics/btp324
50. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA (2010) The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 20(9):1297–303. doi:10.1101/gr.107524.110
51. Albers CA, Lunter G, MacArthur DG, McVean G, Ouwehand WH, Durbin R (2011) Dindel: accurate indel calls from short-read data. Genome Res 21(6):961–73. doi:10.1101/gr.112326.110
52. Reumers J, De Rijk P, Zhao H, Liekens A, Smeets D, Cleary J, Van Loo P, Van Den Bossche M, Catthoor K, Sabbe B, Despierre E, Vergote I, Hilbush B, Lambrechts D, Del-Favero J (2011) Optimized filtering reduces the error rate in detecting genomic variants by short-read sequencing. Nat Biotechnol 30(1):61–8. doi:10.1038/nbt.2053
53. Lassus H, Sihto H, Leminen A, Joensuu H, Isola J, Nupponen NN, Butzow R (2006) Gene amplification, mutation, and protein expression of EGFR and mutations of ERBB2 in serous ovarian carcinoma. J Mol Med (Berl) 84(8):671–81